Untargeted LC-QTOF (ESI +) MS Analysis of Small Serum Metabolites Related to Prostate Cancer and Prostate Specific Antigen

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Abstract

Prostate cancer has an increasing incidence and there is an urgent need for development of new serum biomarkers for early diagnostic as the ones known are ineffective. The aim of the study was to use untargeted metabolomics in order to identify and characterize small metabolite fingerprints in patients with normal *vs* pathologic values of PSA (previously determined by electrochemiluminiscence).

A cohort of one hundred patients with different Prostate Specific amtigen values were investigated by untargeted metabolomics. The serum small metabolite profile determined by high performance liquid chromatography coupled with mass spectrometry, LC-QTOF(ESI$^+$)MS in order to identify specific biomarkers, for normal patient group (PSA = 0-4 ng.ml) and four pathologic groups, having PSA values from 4 to >1000 ng/ml.

The major molecules identified in the samples were polar phospholipids, maily lysophosphatidyl choline derivatives, having m/z values from 496 to 524, like LPC(O-16:0/O-1:0), LPC(18:1/2:0) or PS(18:1(9Z)/0:0), LPC(18:2(9Z,12Z)/0:0 and their isomers and LPC(O-18:1(11Z)/2:0), respectively. Also, small molecules (free fatty acids and prostaglandin derivatives) were identified and are significantly different in pathologic *vs* normal serum samples. Generally the pathologic samples had increased concentrations of all above mentioned molecules. The Principal Component analysis showed , by plot and loadings scores, significant clustering of normal *vs* pathological groups.

Keywords: *prostate specific antigen, small metabolites, untargeted metabolomics, high performance liquid chromatography, mass spectrometry*

INTRODUCTION

Prostate cancer - the most frequently diagnosed tumor in males - is a main cause of morbidity and mortality and its incidence is expected to increase as the population ages. Estimated new cases and deaths from prostate cancer in the United States in 2013 were: new cases: 238,590 and deaths: 29,720 (Tessitore *et al.,* 2013). Prostate cancer is treatable by excision if detected at an early enough stage. However, early diagnosis is hampered by the lack of symptoms and markers. Thus, novel diagnostic and prognostic tools for prostate cancer management are urgently needed (Dhanasekaran *et al.*, 2001; Tomlins *et al.*, 2006).

The serum prostate-specific antigen, discovered in 1971 is considered the most important biomarker for detecting, staging and monitoring cancer of the prostate in its early stage (Rao *et*

al., 2008). The TPSA test was originally approved by the FDA in 1986 to monitor the progression of prostate cancer in men who had already been diagnosed with the disease. The prostate-specific antigen (PSA) is a glycoprotein (molecular weight 30,000–34,000 daltons) having a close structural relationship to the glandular kallikreins. It has the function of a serine proteinase (Henttu and Vihko, 1994). The proteolytic activity of PSA in blood is inhibited by the irreversible formation of complexes with protease inhibitors such as alpha-1-antichymo-trypsin, alpha-2-macroglobulin and other acute phase proteins. In addition to being present in these complexes, about 30% of the PSA present in blood is in the free form, but is proteolytically inactive (Prestigiacomo and Stamey, 1995; Zhang *et al.*, 1995). PSA was initially thought to be solely synthesized by epithelial cells of the prostate and thus was used as a biomarker for diagnosing and managing prostate cancer. However, PSA has also been found in a variety of human normal and tumor cell lines and in biological fluids synthesized by numerous cells, although mainly by prostatic epithelial cells (Diamandis and Yu, 1997; Polascik *et al.*, 1999).

The PSA threshold of 4 ng/mL was set according with literature data (Haythorn and Ablin, 2011) and may indicate the value which is acceptable as normal. Values above 4 that were regarded as pathologic, in correlation with the prostate biopsy (Rao *et al*, 2008; Sreekumar *et al.*, 2009).

The main advantage of PSA testing is its superior sensitivity. The main disadvantage of the test is that it is not very specific because common pathological conditions such as benign prostatic hyperplasia and prostatitis can also cause moderately to conspicuously abnormal test results. These false-positive results may lead to further diagnostic evaluation, increasing costs and use of more invasive procedures. Conversely, efforts to prevent such over estimations may result from a higher number of false positives which may lead to delayed treatment for the aggressive cancers (Stacy and Catalona, 2008, Oh *et al.*, 2003).

Current studies have been focused on the identification of new serum biomarkers by mass spectrometry (MS). Improvement of this technology has provided high accuracy to define mass-to-charge ratio (m/z) and to generate high resolution spectra (Pavlou and Diamandis, 2009; Schroder *et al.*, 2000). A metabolomic approach seems a promising challenge, as tumor cells exhibit defined changes in their metabolism, for non-invasive diagnosis and prognostic evaluation of prostate cancer (Prensner *et al.*, 2012). A dispute has been focused on sarcosine also known as *N*-methylglycine, which is an intermediate and byproduct in glycine synthesis and degradation. It was reported to activate prostate cancer cells and to indicate the malignancy of prostate cancer cells when measured in urine (Struys *et al.* 2010). Sarcosine was identified as a differential metabolite that was greatly increased during prostate cancer progression to metastasis and could be detected in urine. This conclusion has been disputed by Jentzmik group, who reported a different result in 2010. After measuring sarcosine levels in urine samples from prostate cancer patients, they concluded that measuring sarcosine in urine fails as a marker in prostate cancer detection and identification of aggressive tumors (Jentzmik *et al.*, 2010). In addition, another report concluded that serum sarcosine is not a marker for prostate cancer (Struys *et al.,* 2010). A review of the literature reached a similar conclusion (Pavlou and Diamandis, 2009).

The aim of the study was to use untargeted metabolomics in order to characterize small metabolite profile and identify specific biomarkers of serum from patients having different levels of PSA in comparison with patients with low ("normal") PSA using advanced techniques such as high performance liquid chromatography coupled with mass spectrometry and data analysis by Principal Component Analysis.

MATERIALS AND METHODS

Patients cohorts

A number of 100 patients were investigated. The collected serum samples were classified in different groups according to their PSA concentration, previously determined. The first group of 50 patients (Normal Group) had the PSA value below 4 ng/ml, while the second group (Pathological group) had all PSA values above 4 ng/ml, up to 1100 ng/ml. Therefore, the pathological group was divided in four subgroups as described in Tab. 1. The mean values of age distribution and PSA values per subgroup were also calculated.

Tab.1. The groups of pacients used in evaluation, dependent of their range of PSA values, mean PSA values and age distribution.

	Normal group (PSA, ng/ml)	Pathological group (PSA, ng/ml)			
PSA values	0-4	4-10	10-100	100-1000	>1000
n	50	24	15	8	3
Mean age ± SD	54±2.35	63±3.65	71.66±3.8	60.12±3.02	62.33±4.30
Mean PSA ± SD	1.009±0.001	3.53±0.32	44.10±2.5	595.5±42.8	1133±144.3

Blood Sample preparation

Blood serum (100 samples) were collected by standard procedures, after a clothing at room temperature, 30 minutes and centrifugation at 1000*g* for 15 minutes.

The PSA values were determined by the routine assay using electrochemiluminiscence and biorection with two mouse monoclonal antibodies, a biotinylated monoclonal anti-PSA antibody 1.5 mg/l and monoclonal anti-PSA antibody labeled with ruthenium complex 1.0 mg/l.

For metabolomics analysis, the serum samples were diluted with methanol for protein precipitation, in a ratio (1:5), then vortexed, ultrasonicated at 4°C for 5 minutes and centrifuged at 15 000*g* for 15 minutes. The supernatant was collected, filtered through 0.2 µm filters and kept in the deep freezer until analysis.

LC–ESI(+)-Q-TOF-MS analysis

Aliquots of 5 µl of each supernatant were subjected to liquid chromatography separation, using a Thermo Scientific HPLC UltiMate 3000 system equipped with a quaternary pump delivery system Dionex UltiMate 3000 (UHPLC+ focused), Acclaim C-18 column (3µm, 2.1 X 50 mm), autosampler and Dionex Ultimate 3000 photodiode array detector. The column temperature was set at 40°C.

The detection was made by mass spectrometry using a Bruker Daltonics MaXis Impact Q-TOF operating in positive ion mode (ESI^+). The mass range was set between 50-1000 m/z. The nebulizing gas pressure was set at 2 bar, the drying gas flow at 8 L/min, the drying gas temperature at 180 ºC. Before each run, a calibrant solution of sodium formate was injected. The control of the instrument and data processing were done using TofControl 3.2 and Data Analysis 4.1 (Bruker Daltonics) softwares.

The mobile phases used were 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The flow rate was set at 0.5 $mL \cdot min^{-1}$. The gradient elution initial conditions were 1% B to 15% B, with a linear gradient from 0 to 3 min, followed by linear gradient to 50% B at 6 min, linear gradient to 95% B at 9 min, isocratic on 95% B for 6 min and then returned to initial conditions at 15 min and kept isocratic on 1% B for 5 min.

Biostatistics

The LC–MS data of both normal and pathological samples were processed using the "Find Molecular Features" (FMF) peak detection algorithm. The major advantage of this processing method is that real signal is differentiated from background noise. FMF compounds were further for PCA analysis.

The Principal Component Analysis (PCA) method was used to discriminate between normal and pathological samples. PCA was performed using the Profile Analysis software (Bruker, Daltonics). The principle behind PCA analysis was to use a bucket table that contained the information regarding the identified compounds in the chromatograms. Thus each bucket was described by two values, retention time and *m/z* value. The given values were the corresponding center values of the retention time range and the *m/z* range in the respective bucket as calculated by advanced bucketing function in Profile Analysis software. The obtained bucket table was further used in PCA analysis to separate patients with normal PSA values of patients with pathological PSA values. Therefore the untargeted metabolomics approach delineated between normal and pathogen samples.

PCA converts a larger number of observed variables into a smaller number of variable (called principal components), that will take in account most of the variance in the observed variables. Groups formed in the two dimensional scores plot indicates the similarities between samples

and allows the discrimination among normal or pathological samples.

RESULTS AND DISCUSSIONS

Preliminary evaluation of patients PSA status

Table 1. Includes the mean values of PSA obtained for each age category of pacients. The PSA values from 0 to 4 ng/ml were considered normal, while the PSA values between 4-10 ng/ml were considered to characterize benign prostate hyperplasia or prostatitis (Hankey *et al.*, 1999). The PSA values higher than 10 ng/ml were considered to be caused by prostate cancer, at different stages. In case of the pathological subgroups (4-10, 10-100, 100-1000, >1000 ng/ml) the 4 subclasses of patients, had the PSA average values of 3.53±0.32 (for the mean age group of 63 years) , 44.10±2.5 (for the mean age group of 70 years), 595.5±42.8 (for the mean age group of 60 years) and 1133±144.3 ng/ml (for the mean age group of 62 years).

Within the studied cohort, the average age for the high pathological subgroups (PSA > 100) group was 60-62 years, while for the moderate pathological samples, 71 years. It is suggested that people aged more than 55 years may represent a risk group, as it was also confirmed previously (Hankey *et al.,* 1999; Lokhov *et al.*, 2010).

LC-QTOF (ESI +) MS analysis

The comparative base peak chromatograms (BPC) of a typical normal *vs* pathological serum sample is shown in Fig. 1 A and B, respectively. A number of 14 main peaks were identified for normal serum and 20 main peaks for the pathological sample. Peak identification was made according to their retention times, the released ions of protonated molecules $[M + H]^+$ and literature data as summarized in Tab.2.

According to Fig. 1 A and B, as well to Table 1, it is obvious that the major peaks separated corresponded mainly to lyso-derivatives of Phosphatidyl choline, with different fatty acids (marked as boded in the table) and their isomers. Integral PC molecules and free fatty acids were also identified in this methanolic extracts of serum.

Biostatistics

As illustrated in Fig. 2, the PCA analysis generated comparisons of PC1 and PC2 accounted 97.5% of the total variance, considering the scores (based on score plots).

The score plot shows the grouping of samples according to the grouping of PSA levels in normal and pathologic ones. A great variation was observed inside the pathological groups. No significant

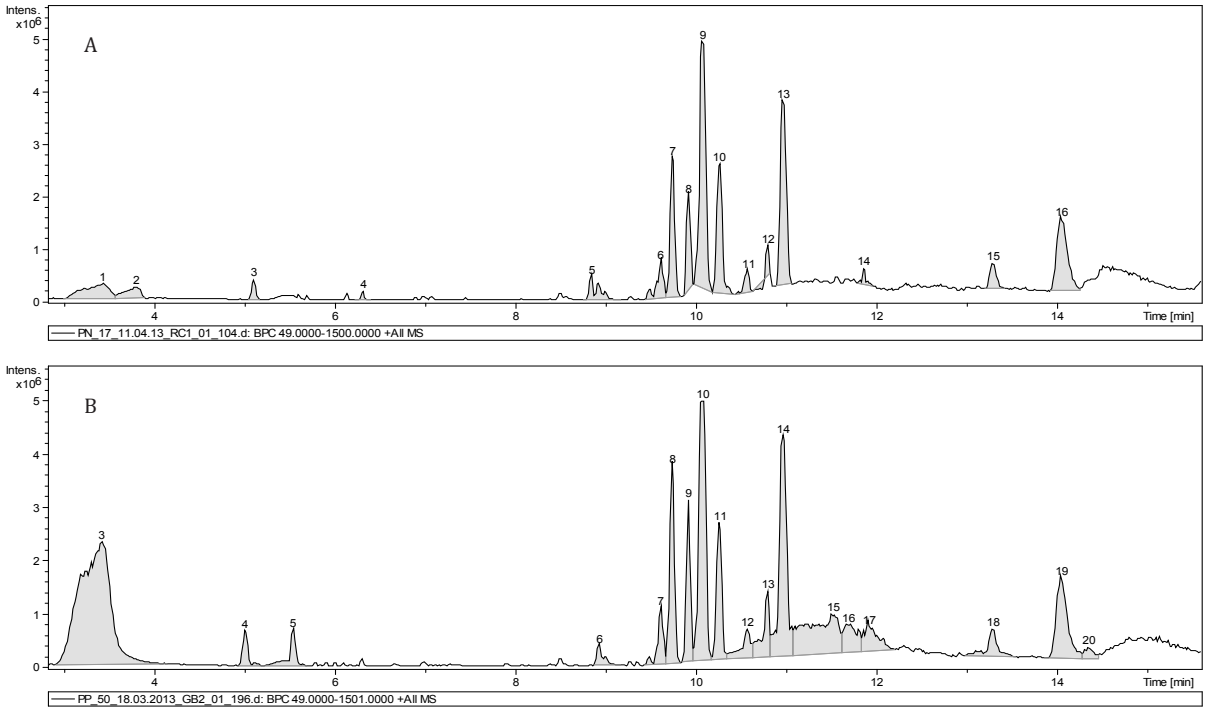


Fig.1. Comparative base peak chromatograms of a typical normal serum with PSA<4ng/ml (A) and a pathologic serum with PSA=728 ng/ml(B).

Tab. 2. Identification of main peaks in normal and pathological serum samples, based on m/z data and tentative assignment of the metabolites , using Lipid Maps Bank (www.lipidmaps.org/tools/ms/LMSD_search)

Normal Patient (PSA<4)				Pathologic patient (PSA=728ng/ml)			
Peak number	tR	$[M + H]^+$ *m/z*	Tentative assignment of the compound	Peak number	tR	$[M + H]^+$ *m/z*	Tentative assignment of the compound
3	5.1	188.0706	n-Decanohydroxamic acid	4	5	188.0757	n-Decanohydroxamic acid
4	6.3	313.1569	Arachidonic acid	5	5,6	246.1309	Pentadecylic acid
5	8.9	324.1399	Anandamide (18:2, n-6	6	8.9	177.0591	6,8-dihydroxy-octanoic acid *Or* 3-propylmalic acid
6	9.6	520.3453	LPC(18:2(2E,4E)/0:0)	**7**	**9.6**	**520.3552**	**LPC(18:2(2E,4E)/0:0)**
7	**9.7**	**520.3457**	**LPC(18:2(9Z,12Z)/0:0**	**8**	**9.7**	**520.3552**	**LPC(18:2(9Z,12Z)/0:0)**
8	**9.9**	**496.3452**	**LPC(O-14:0/2:0)**	**9**	**9.9**	**496.3556**	**LPC(O-16:0/O-1:0)**
9	**10.1**	**496.3457**	**Isom.LPC(O-14:0/2:0)**	**10**	**10.1**	**496.3558**	**Isom.LPC(O-16:0/O-1:0)**
10	**10,3**	**522.3615**	**LPC(O-18:1(11Z)/2:0)**	**11**	**10,3**	**522.3718**	**LPC(O-18:1(11Z)/2:0)**
11	**10,6**	**149.0225**	**Mevalonic acid**	12	10,6	149.026	Mevalonic acid
12	**10,8**	**524.3769**	**LPC(O-16:0/2:0)**	**13**	**10.8**	**524.3876**	**LPC(16:0/2:0)**
13	**11**	**524.3775**	**Isom.LPC(O-16:0/2:0)**	**14**	**11**	**524.3835**	**Isom.LPC(16:0/2:0)**
14	11.9	313.2762	Eicosanoic acid	15	11.5	760.6042	PC(16:0/18:1(9Z))
15	13.3	391.2872	5,9,17-hexacosatrienoic acid	16	11.7	758.5924	PC(16:0/18:2(10E,12Z))
17	14.5	782.5818	PC(16:0/20:4(5Z,8Z,11Z,14Z))	17	11.9	758.5923	PC(16:0/18:2(10E,12Z))
				18	13.3	391.2955	5,9,17-hexacosatrienoic acid

discriminations between groups of 4-10 and 10-100 PSA ranges, but slight discrimination against the most pathological group (100-1000), located at lower PC2 values. The samples with PSA > 1000 ng/ml were considered outliers and excluded from the PCA model. The normal group presented high homogeneity since almost all samples were clustered together in two groups.

The compounds identified by FMF method, which contributed to sample differentiation are highlighted in the loadings plot (Fig. 3).

The distribution of samples was made almost exclusively along PC1 axis. One can see that molecules having m/z values of 496.3558, 524.389, 520.358 and 522,374 are mainly responsible for the discrimination. These molecules are lysophosphatidyl choline derivatives like LPC(O-16:0/O-1:0), LPC(18:1/2:0) or PS(18:1(9Z)/0:0), LPC(18:2(9Z,12Z)/0:0 and their isomers and LPC(O-18:1(11Z)/2:0), respectively .

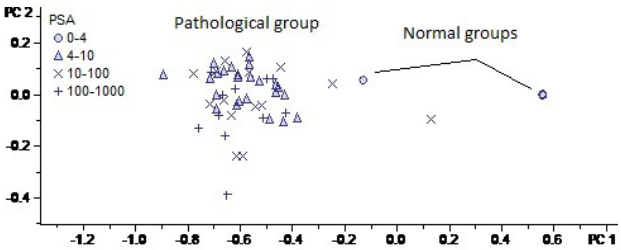


Fig.2. The score plot of the PCA analysis of MS data from serum samples : 50 normal PSA values and 50 pathological values (clustered in 4 groups, as presented in the figure).

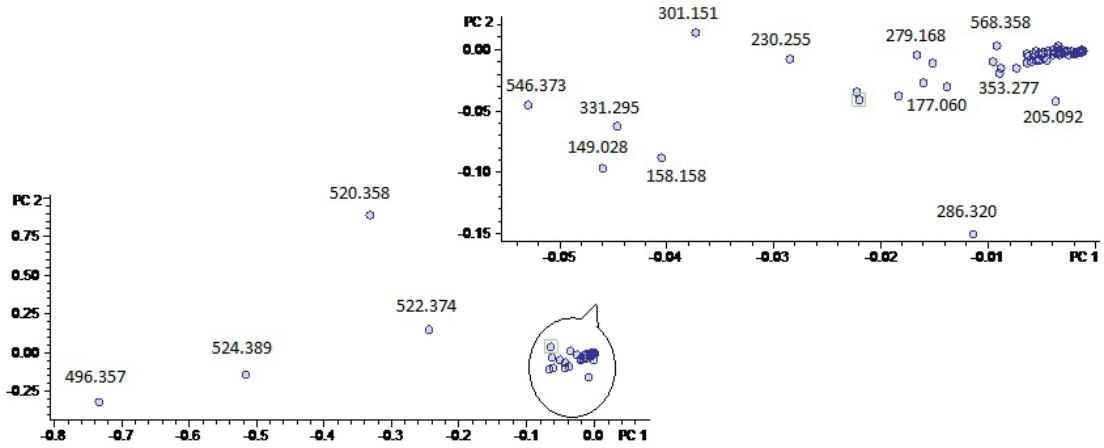


Fig. 3. PCA loading plot (3). The numbers written on the loading plots are representing the FMF LC-MS peaks (m/z). Representative statistics plot of the selected loadings are detailed in Tab.3.

Table 3 shows the bucket statistics plot of the selected loadings. There were selected the most significant examples of loadings with different production patterns in major and minor metabolites, indicating the relative production of each specific metabolite and their influence on group's formation in the score plots. This kind of representation are useful to see the significance of differences between the normal group and the pathological groups for each biomarker.One can notice that major molecules belonging to lysophospholipid class, having m/z values from 496 to 524 were significantly increased in pathological samples. Meanwhile, also small molecules, such as free fatty acids and prostaglandin derivatives were identified and are significantly different in pathologic *vs* normal serum samples. Generally the pathologic samples had increased concentrations of such small molecules.

Our data are in agreement with previous findings which identified phosphocholine-containing lipids as individual lipid biomarkers for prostate cancer and 15 apparent lipid species were considered (Rao *et al.,* 2008).

Meanwhile, Zhao *et al* (2007) noticed decreased plasma levels of several lysophosphatidylcholines (LPCs), including 18:1- and 18:2-LPC, in colon-rectal cancer patients compared with controls ($P < .001$), as mentioned by

This result might suggest up-regulation of phosphocholine metabolism in patients with prostate cancer, and is consistent with previous findings that high grade prostate cancer tissues had higher concentration of phosphocholine derivatives as compared with low grade prostate cancer (Zhou *et al.,* 2012). Another study showed that three out of 13 lipid classes, phosphatidylethanolamine (PE), ether-linked phosphatidylethanolamine (ePE) and ether-linked phosphatidylcholine (ePC) could be considered as biomarkers in diagnosis of prostate cancer (Da Costa *et al.,* 2012).

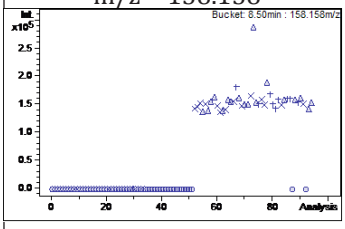
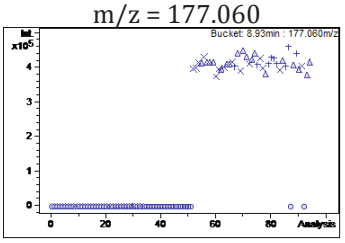
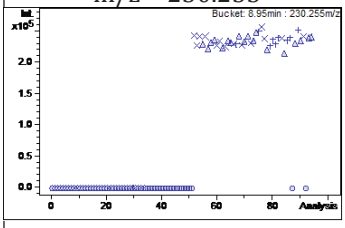
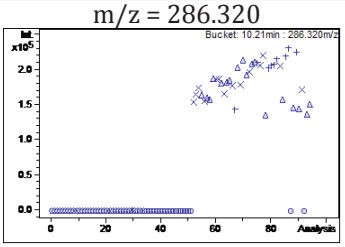
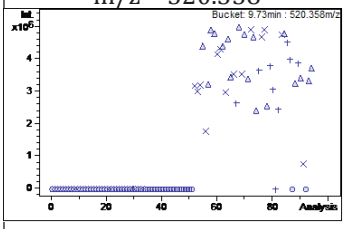
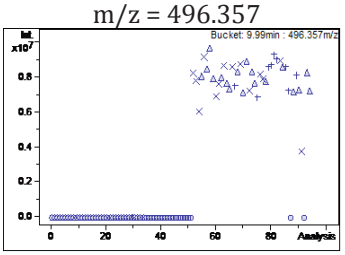
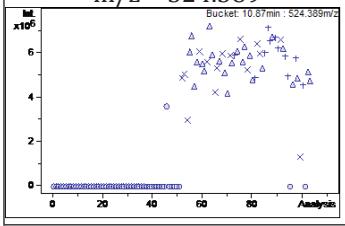
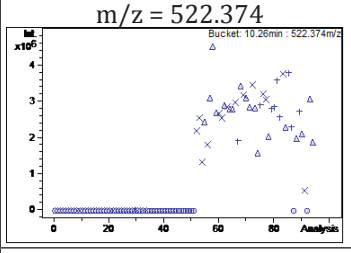
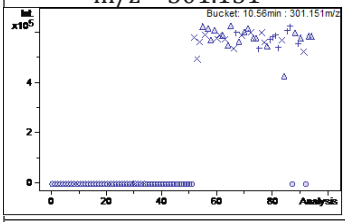
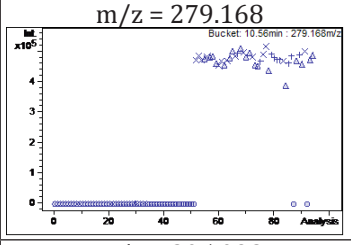
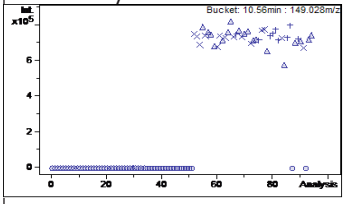
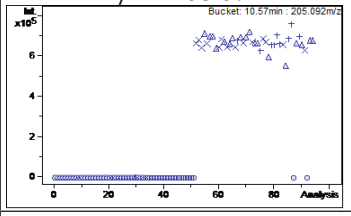
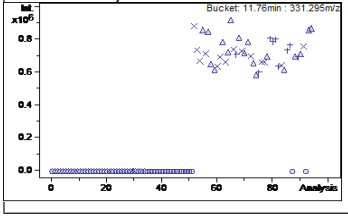
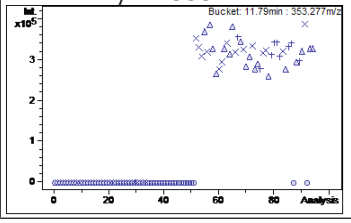
CONCLUSION

A cohort of 100 patients with different Prostate Specific amtigen values were investigated by untargeted metabolomics. The serum small metabolite profile determined by LC-QTOF(ESI$^+$)MS allowed the identification of specific biomarkers, for normal and four pathologic groups, havinf PSA values from 4 to >1000 ng/ml.

The major molecules identified in the samples were lysophospholipids, maily Phosphatidyl choline derivatives, having m/z values from 496 to 524, like LPC(O-16:0/O-1:0), LPC(18:1/2:0) or PS(18:1(9Z)/0:0), LPC(18:2(9Z,12Z)/0:0 and their isomers and LPC(O-18:1(11Z)/2:0), respectively. Also, small molecules (free fatty acids and prostaglandin derivatives) were identified and are significantly different in pathologic *vs* normal serum samples. Generally the pathologic samples had increased concentrations of all above mentioned molecules.

Further investigations are needed to use the targeted metabolomics in order to identify alterations of such polar lipid species in normal serum

Tab. 3. Representative statistics plots of PCA score loadings with influence in sample grouping , showing the differences between the normal group and the pathological groups for each biomarker.

Bucket statistic	Tentative identification	Bucket statistic	Tentative identification
m/z = 158.158 	2-amino-2,4-hexadienedioic acid	m/z = 177.060 	6,8-dihydroxy-octanoic acid *Or* 3-propylmalic acid
m/z = 230.255 	Amino-tridecanoic acid	m/z = 286.320 	C17 Sphingosine
m/z = 520.358 	LPC(18:2(2E,4E)/0:0)	m/z = 496.357 	LPC(O-16:0/O-1:0)
m/z = 524.389 	LPC(16:0/2:0)	m/z = 522.374 	LPC(O-18:1(11Z)/2:0)
m/z = 301.151 	(S)-3-hydroxystearic acid *Or* 8,11,14,18-Eicosatetranoic acid *Or* 5,8,11-Eicosatrienoic acid	m/z = 279.168 	Alpha/ gamma/ -linolenic acid
m/z = 149.028 	Dihydroxy-fumaric acid *Or* Mevalonic acid	m/z = 205.092 	3,10 Dihydroxydecanoic acid
m/z = 331.295 	4,7,10,13,16-docosapentaenoic acid *Or* 9,12,13-TriHOME (trihydroxyoctadecenoic acid)	m/z = 353.277 	Prostaglandins E2/ D2/H2 (9-oxo-11R,15S-dihydroxy-5Z,13E-prostadienoic acid)

and in a larger group of confirmed prostate cancer samples, in order to identify specifically the reliable lipid markers and their concentrations and dynamic during cancer development, associated with the pathological phenotypes of prostate cancer.

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